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SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE		AD-A213 016	Form Approved OMB No. 0704-0188
1a. REPORT SECURITY CLASSIFICATION	N/A		N/A
2a. SECURITY CLASSIFICATION AUTHORITY	N/A	3. DISTRIBUTION/AVAILABILITY OF REPORT	N/A
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE	N/A		N/A
4. PERFORMING ORGANIZATION REPORT NUMBER(S)	N/A	5. MONITORING ORGANIZATION REPORT NUMBER(S)	N/A
6a. NAME OF PERFORMING ORGANIZATION: Johns Hopkins University	6b. OFFICE SYMBOL (If applicable): 5L406	7a. NAME OF MONITORING ORGANIZATION: Office of Naval Research	
6c. ADDRESS (City, State, and ZIP Code): Baltimore, Maryland 21205		7b. ADDRESS (City, State, and ZIP Code): Washington, D.C. 20036	
8a. NAME OF FUNDING/SPONSORING ORGANIZATION: Office of Naval Research	8b. OFFICE SYMBOL (If applicable): N 00014	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER	
8c. ADDRESS (City, State, and ZIP Code): Arlington, Virginia 22217-5000		10. SOURCE OF FUNDING NUMBERS: PROGRAM ELEMENT NO: N00014; PROJECT NO: 87; TASK NO: K; WORK UNIT ACCESSION NO: 0503	

11. TITLE (Include Security Classification)

Investigation of a Novel Serotonin Receptor System in the Mammalian Brain

12. PERSONAL AUTHOR(S)

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13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Final	FROM 03/87 TO 03/89	1989, 06, 27	9

16. SUPPLEMENTARY NOTATION

17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	Serotonin receptors, Neurotoxicity, Blood brain, Barrier, Psychomimetic drugs.

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

In the period of this award, we found that $5HT_{1C}$ receptor in the pig choroid plexus couples not only to increased phosphatidylinositol turnover but also to increased production of cyclic GMP. Cyclic GMP but not cyclic AMP appears to play a role in the negative feedback mechanism with respect to serotonin-induced phosphatidylinositol turnover.

(Continue on reverse side.)

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION	
☐ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT ☐ DTIC USERS		
22a. NAME OF RESPONSIBLE INDIVIDUAL: Fusao Hirata, M.D., Ph.D.	22b. TELEPHONE (Include Area Code): (301) 955-3291	22c. OFFICE SYMBOL: 5L406

DD Form 1473, JUN 86 — Previous editions are obsolete — SECURITY CLASSIFICATION OF THIS PAGE

S/N 0102-LF-014-6603

89 7 31

Recent progress in molecular biology of the serotonin receptors by several laboratories including ours has shown that $5HT_{1C}$ receptor has high homology to $5HT_2$ receptor and that it is distinct from $5HT_{1a}$ receptors. The both the $5HT_{1C}$ and $5HT_2$ receptors couple to phosphatidylinositol turnover, while $5HT_{1a}$ receptor couples to adenylate cyclase. Because of similarities in the structural and pharmacological properties between $5HT_{1C}$ and $5HT_2$ receptors in the brain, studies on the distribution of the receptors using cDNA clones and radioactive ligands may not provide confirmatory results. To conquer these problems, we have attempted to raise antibodies against several epitopes of $5HT_{1C}$ receptor, whose peptide sequences are predicted form its nucleotide sequences. These antibodies immunoprecipitated a single protein with an apparent molecular weight of 56,000 from the choroid plexus of rats, mice and pigs but not from tissues where no apparent $5HT_{1C}$ receptor exists as measured by ^{125}I LSD binding.

The primary cultured choroid plexuses from rats and pigs actively uptook Cd^{2+} without changes of viability. Serotonin had no effects on this uptake. Cd^{2+} can replace Zn^{2+}, a crucial metal in the DNA binding proteins (transcriptional regulators). Since Cd^{2+} can also replace other divalent cations, we predict that accumulation of this heavy metal in the epithelial cells will cause some functional changes of the choroid plexus, especially transport systems. Alcohol preferentially inhibited the production of cyclic GMP elicited by serotonin, but less affected the turnover of phosphatidyl-inositol. To investigate the consequences of exposure of epithelial cells to these toxic agents in vitro, we have established cell lines of epithelial cells from normal choroid plexus and murine choroid plexus tumors.

Together with cDNA probes (specific to $5HT_{1C}$ receptor) and anti-peptide antibodies against $5HT_{1C}$ receptor which our laboratory has recently developed, choroid plexus epithelial cell lines will make it possible to study effects of neurotoxic agents on expression of the receptor gene as well as on the signal transduction mechanism, both of which, in turn, alter the epithelial cell function, ion fluxes. These informations will be crucial to better strategies for defense against neurotoxic agents and for treatment of victims of such exposure.

Introduction:

Serotonin receptors control important biochemical and physiological processes in both the central and peripheral nervous systems. These receptors are associated with tryptamine-induced clonic seizures, serotonin behavioral syndrome, contraction of smooth muscle, action of antidepressant, and action of hallucinogenic drugs (see reviews 1,2). Serotonin receptors are now classified into several subtypes mainly by radioligand binding studies. Among those serotonin receptor subtypes, $5HT_{1C}$ and $5HT_2$ receptors are now known to couple to increased turnover of phosphatidylinositol as a second messenger system, and are closely related with respect to pharmacological characteristics. $5HT_{1C}$ receptor is mainly located in the choroid plexus, while $5HT_2$ receptor resides in layer IV cortex. Distinct from $5HT_2$ receptor whose functions are known to induce tryptamine seizure, vascular contraction and head twitches, the functions of $5HT_{1C}$ receptor are not yet known. Some evidences suggest that it reduces cerebrospinal fluid production in the choroid plexus by opposing beta-adrenergic stimulation (3,4). These receptors are proposed to regulate ion fluxes through $Na^+/K^+/Cl^-$ cotransport, Na^+/K^+-ATPase, Ca^{2+} dependent K^+ channel and Cl^- channel as well as transport of hormones and neurotransmitters.

The choroid plexus consists blood-cerebrospinal fluid (blood-brain) barriers and functions as selective transport system between blood and brain (5,6). Since various neurotoxic agents have to pass through this barrier, we presumed that the choroid plexus might be the first target of these neurotoxic agents. Accordingly, agents which affect $5HT_{1C}$ and $5HT_2$ receptor functions in the brain should have some effects on $5HT_{1C}$ receptor in the choroid plexus. Monitoring such effects on $5HT_{1C}$ receptor in the choroid plexus will provide us with a better understanding of the mechanism of toxicity of these compounds. The objectives of our proposed research were to study the physiological and metabolic consequences of activation of a newly discovered brain neurotransmitter receptor system, the serotonin $5HT_{1C}$ receptor system which was found to be abundant in the choroid plexus. To approach to this goal, we proposed to investigate the relationship between $5HT_{1C}$ receptor and other mammalian brain serotonin receptors with regard to the detailed molecular consequences of activation of phosphoinositide turnover. Our ultimate goal is to provide a better understanding of neurotoxic action at a variety of brain serotonin receptor sites, using $5HT_{1C}$ receptor in the choroid plexus as a model system, because the choroid plexus is the major route from blood to brain where neurotoxic agents are delivered across (5,6).

Progress Reports:

1. Distribution of 5HT receptor in the brain.

Our laboratory has been engaged in development of ^{125}I-LSD and ^{125}I-MIL as selective, high affinity labels for serotonin receptors. Paul Hartig in our laboratory investigated in collaboration with Dr. M. Molliver's laboratory on mapping serotonergic innervation and serotonin $5HT_2$ receptors in the neocortex. $5HT_2$ receptor was measured with ^{125}I-MIL (2,000 Ci/mmol) as a high affinity (Kd=0.14 nM) ligand. To block $5HT_{1C}$ receptor binding sites, this ligand was used in the presence of specific $5HT_{1C}$ receptor blocking agents. Further, we used fine grain Kodak AR10 stripping film to obtain a high resolution autoradiography. Serotonergic innervation was mapped by immunohistochemistry with an antiserum against serotonin. In over mapping studies, we found that regional variations in the density of $5HT_2$ receptors are matched by similar

regional variations in the distribution of serotonin axons in most forebrain areas. In the somatosensory cortex, the $5HT_2$ receptor were localized to upper layer Va, which lies in contrast tot most previous studies which had localized these sites to layer IV based on lower resolution mapping with less precise techniques for layer matching. The $5HT_2$ receptor sites in layer Va were in precise register with a dense plexus of fine varicosity serotonin axons arising from the dorsal raphe nucleus. These studies indicate a close correlation between receptor sites and innervation, especially with respect to the anatomical linkage between $5HT_2$ receptors and a subset of serotonergic fibers arising from the dorsal raphe nucleus. Serotonin $5HT_{1c}$ receptor in the choroid plexus was found to be apically located (in the cerebrospinal fluid side of epithelial cells). Although substantial cholinergic and adrenergic innervation is found from the vagal and glossopharyngeal nerves, serotonergic fibers are not directly innervated to this tissue. Supraependymal 5HT fibers are the primary source of CSF-borne serotonin that activates and regulates $5HT_{1c}$ receptor in the choroid plexus. The damages of these fibers by the treatments with 5.7-DHT (dihydroxytryptamine) or MDMA (3.4-methylenedioxymetanphetamine) resulted in upregulating $5HT_{1c}$ receptor as measured by ^{125}I-LSD binding (see Publication 2,6,7).

2. The second messenger system of 5HT receptor in the choroid plexus.

Following our previous findings that serotonin receptor in the choroid plexus is $5HT_{1c}$ subtype coupling to the phosphoinositide turnover, we tested the effects of various serotonergic ligands upon the phosphoinositide turnover using the pig choroid plexus. The tissue was cut into 350 um^2 sections and incubated with 1uCi/ml ^{3}H-myoinositol in gassed ($95\%O_2/5\%CO_2$). Krebs Ringers buffer pH 7.4, containing 108 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 0.9 mM KH_2PO_4, 25 mM $NaHCO_3$, 10 mM glucose, 2.5 mM $CaCl_2$, 10 mM LiCl, and 10 uM pargyline for 1hr at 37°C. The reactions were run as indicated and terminated by adding chloroform/methanol (1/2, v/v). After centrifugation at 10,000 x g for 5 min by a bench top centrifuge, the supernatants were analyzed by the stepwise chromatography on Dowex 1X8 (200 meshes) as described. Three groups of compounds were tested for their abilities to stimulate phosphoinositide turnover, and their affinities (concentrations required for half maximal response) and efficacies (degrees of maximal response) were compared. The relative efficacies were indolalkylamines > phenylpiperazines > ergolines. The ergoline LSD possessed the highest affinity with minimal efficacy and bromination at the 2 position of LSD completely abolished efficacy. These observations showed that $5HT_{1c}$ receptors are also the sites where various psychotropic drugs act (Publication 14).

Stimulation of the pig choroid plexus with serotonin also resulted in increased production of cyclic GMP. This increase could be inhibited by ketanserin and mianserine but not by spiperon, suggesting that this response is also mediated though $5HT_{1c}$ receptor. In addition, verapamil, a calcium channel blocker, and low calcium buffers (below 100 uM) decreased cyclic GMP production. Neomycin (100 uM) reduced cyclic GMP by 30%, whereas p-bromophenacylbromide (100 uM), an inhibitor of PLA_2, decreased it by 60%. Although indomethacin, an inhibitor of cyclooxygenase, enhanced cyclic GMP production by 60%, BW-755C, an inhibitor of lipooxygenase completely blocked cyclic GMP formation. These results clearly demonstrated that activation of PLA_2 causes release of arachidonic acid, which in turn is metabolized into lipooxygenase products to activate guanylate cyclase. At the present, we do not know whether PLC and PLA_2 activations are sequential or separate events, nor which second messenger, arachidonate, cyclic GMP or diacylglycerol plus phosphates is

responsible for regulation of ion fluxes. However, sodium nitroprusside, a guanylate cyclase activator and ANF, a ligand which directly activates the particulate form of guanylate cyclase, significantly decreased serotonin-elicited phosphoinositide turnover. These observations suggest that cyclic GMP provides a negative signal to the serotonin receptor system (Publication 12,13).

Taken together, the above observations suggest that after stimulation with serotonin, the choroid plexus produces Ca^{2+}, diacylglycerol, arachidonate plus its metabolites and cyclic GMP as the intracellular messengers. Further, the choroid plexus is composed of not only epithelial cells but also endothelial cells and fibroblasts. Because of this cellular heterogeneity, the observed events might take place in cells other than epithelial cells. Therefore, we decided to carry out isolation of epithelial cell lines next rather than to study ion fluxes using this heterogous cell populations. When we establish epithelial cell lines and the signal transduction system of $5HT_{1c}$ receptor, it would be easier to study the consequence of $5HT_{1c}$ receptor activation regarding to epithelial cell functions.

3. cDNA cloning of $5HT_{1c}$ receptor in the murine choroid plexus tumors.

A strain of transgenic mice carrying SV40 early region genes develops spontaneously large choroid plexus epithelial tumors (100-300 mg/mouse). We found that these tumors are an extremely rich source of the $5HT_{1c}$ receptors (6.6 pmol/mg protein) with binding properties in good agreement with previous $5HT_{1c}$ receptor. We have now established a stable colony of these mice at the Johns Hopkins University, School of Hygiene and Public Health, and have been able to obtain several grams of these tissues for further study. In collaboration with Drs. Norm Davidson and Henry Lester at Cal Tech Inst, Beth Hoffman and Paul Hartig in our laboratory had participated in cloning cDNA encoding $5HT_{1c}$ receptor of murine choroid plexus tumors. The strategy of cloning involved the use of hybrid depletion techniques to screen a cDNA library for the $5HT_{1c}$ receptor. This novel approach has been published (Publication 1). Briefly, mRNA from the murine choroid plexus could express $5HT_{1c}$ receptor, when they were injected into xenopus oocytes. This expressed receptor stimulated Cl^- flux via phosphatidylinositol system in oocytes; thus, mRNA expression could be detected by Cl^- flux by the electrode without the need for purification and sequencing of the receptor. When cDNA-mRNA hybrids that contained $5HT_{1c}$ receptor mRNA were excluded, the remaining mRNA could not express $5HT_{1c}$ receptor. From 1,200 cDNA clones tested, one clone (D9) was identified as $5HT_{1c}$ receptor. Although the previous study had shown that $5HT_{1c}$ receptor is a single subunit protein encoded by a 5Kb RNA, D9 was 1.2Kb long. We rescreened cDNA library and isolated one clone (clone 157) with 4Kb. This clone appeared to contain all 3'- region but 5'- end terminated within the protein coding region. A group from the Columbia University has used a modification of this cloning strategy and from 1.2 million clones, obtained cDNA which covers the entire region of rat choroid plexus $5HT_{1c}$ receptor. This Columbia clone contained a single open reading frame encoding aprotenin of 460 amino acids with a molecular weight 52,000. The general properties of this protein were discussed in "Significance". Comparison of our clone 157 to the Columbia's one suggests that a 95 base pair deletion has occurred in our clone as shown in Fig. 1. The black circles represents amino acids that are missing from our clone and striped circles are the seven amino acids out of 357 which differ between the two clones. This deletion may occur by a cloning artifact(s) and/or by incorrect splicing of precursor mRNA in the tumor cells. In order to complete the 5'- coding region of the receptor and to explore the gene

structure. Hoffman and Hartig in our group together with Drs. Lester and Davidson in Cal Tech. have focused on genomic clones for $5HT_{1C}$ receptor and discovered the presence of unexpected restriction fragments on high stringency Southern blots of rat genomic DNA. These findings suggest the possible presence of introns in the 5'- coding region of $5HT_{1C}$ receptor. This is a surprising finding, since all G protein coupled neurotransmitter receptor have been reported to have no introns. However, there are some cases in which several G protein coupled receptors such as rhodopsin contain introns in their 5' and 3' untranslation regions.

4. Production of antipeptide antibodies against $5HT_{1C}$ receptor.

Since the cDNA clones isolated from mice and rats have high homology in their amino acid sequences (only 7 amino acids out of 357 amino acids are different), we decided to raise antipeptide antibodies against $5HT_{1C}$ receptors. Four peptide epitopes (amino acids 41-51, 292-301, 433-442 and 451-460) were chosen. A cysteine residue was added to either N- or C- termini of these peptides and then, the peptides were conjugated to KLH by the S-S linkage. The peptide-KLH complexes were immunized into rabbits. Antipeptide antibodies were purified by the ammonium fraction followed by affinity chromatography on KLH-coupled agarose and protein A-coupled agarose. These antibodies immunoprecipitated a single protein with an apparent molecular weight of 56 K as judged on SDS-PAGE, among ^{125}I-Bolton Hunter labeled proteins solubilized from the pig, mouse and rat choroid plexuses by 0.1% triton, 0.1% Nonidet P40 or 0.1% CHAPS. Preimmune sera nor antibodies treated with peptides didn't immunoprecipitated this protein. Therefore, we concluded that these antibodies are specific to $5HT_{1C}$ receptor. This was further supported by our findings that these antibodies do not bind to the tissues and cells which do not contain $5HT_{1C}$. Nevertheless, the antibodies did not inhibit ^{125}I-LSD binding. Further, we have not yet succeeded in demonstrating that the protein isolated by the antibodies and protein A-beads have ^{125}I-LSD binding capacity, although we have tried to reconstitute it with phospholipid vesicles. In collaboration with Drs. M. Kuhar and E. DeSouza of the NIDA, we are now investigating whether these antibodies can stain $5HT_{1C}$ receptor in the rat brain. If the distribution of $5HT_{1C}$ receptor immunostained with these antibodies are identical to that examined with ^{125}I-LSD, it will be a stronger evidence that these antibodies are specific to $5HT_{1C}$ receptor.

5. Production of epithelial cell lines.

We dissected tumors and dissociated cells with 1 mg/ml collagenase. After centrifugation (3,000 rpm) on a Ficoll gradient (8, 10, 12% and 15%) we isolated epithelial cells between 12 and 15% Ficoll and cultured in RPMI 1640 or DMEM media containing 10% FCS, 5u/ml PC and 5ug/ml SM. Contaminated fibroblasts and endothelial cells were excluded by allowing the cells overnight to attach a petri dish. The suspended cells were cultured in defined media without fetal calf serum. DMEM/Ham F12 (1/1,v/v) contained 5 ug/ml transferrin, 3 ug/ml insulin, 0.25 ug/ml EGF, 20 nM cortisone, 5 nM thyroxin and 3 nM sodium selenite supplemented with 5 unit/ml penicillin and 5ug/ml streptomycin. When Ca^{2+} concentration was lowered to 50 uM or when the plates were coated with collagen, the epithelial cells were attached to petri dish. Otherwise, the cells glowed as suspended cells. Interestingly, addition of 1 uM serotonin appeared to enhance proliferation of the epithelial cells. We have maintained these cell lines more than 6 month, and are now characterizing the properties of these cell lines. The cells express approximately 3 pmol/mg protein of ^{125}I-LSD binding sites. Therefore, we believe that this cell line

will serve as a good model to study regulation of receptor-effector coupling system of $5HT_{1C}$ receptor.

6. Toxicology of $5HT_{1C}$ receptor in the choroid plexus.

Cd^{2+}, Hg^{2+} and other heavy metals are known to affect various neurological functions. These metals are actively accumulated in epithelial cells of the choroid plexus (7). When rat choroid plexus were sliced and cultured in Krebs-Ringers solution together with $^{109}Cd^{2+}$, they uptook $^{109}Cd^{2+}$ in a time dependent manner. However, the uptake of $^{109}Cd^{2+}$ was not affected by serotonin nor its antagonists, mianserin and ketanserin.

Serotonin stimulated the formation of cyclic GMP as described above. Ethanol inhibited it. A concentration required for half maximal inhibition was around 0.06% (v/v). In contrast, ethanol had no effects on the phosphoinositide turnover at this concentration range. Since it also reduced the cyclic GMP production elicited by sodium nitroprusside, we concluded that ethanol directly inactivates a soluble form of guanylate cyclase. Cyclic GMP is proposed to reduce the cerebrospinal fluid (CSF) production. Hence, ethanol might have some effects on the CSF production in vivo (Abstract 2).

Prospects for future study:

Receptor coupled ion channels and/or transporter systems are targets of various neurotoxicants including heavy metals, alcohols and other organic solvents, to which personnel in the navy are vulnerable. Among these compounds, Cd^{2+} is known to be accumulated in the epithelial cells of the choroid plexus (7). This cation replaces Ca^{2+} in the intracellular storage, thus leading to enhance phosphoinositide turnover. However, it inhibits protein kinase C (8). Serotonin $5HT_{1C}$ receptor in the choroid plexus couples to increased turnover of phosphoinositide and after all, regulates ion fluxes mediating through activation of protein kinase C. In this regard, it is obvious that Cd^{2+} may compete with action of serotonin. Epithelial cells in the choroid plexus are also responsible for transport of hormones and neurotransmitters such as thyroxin and catecholamines. It is not known whether serotonin can enhance or inhibit such transport.

$5HT_{1C}$ receptor is located not only in the choroid plexus but also in the cortex. $5HT_2$ receptor and $5HT_{1C}$ receptor have good association with subtypes of serotonergic neurons with fine and coarse varicosities. Since fine varicosity serotonergic fibers projected from Raphe nuclei is recently reported to be more sensitive to a neurotoxic agent. Furthermore, $5HT_{1C}$ receptor and $5HT_2$ receptor have the similar pharmacological profiles and primary structures. All these observations implicate importance of $5HT_{1C}$ receptor in the choroid plexus as a model system to study the mechanism of neurotoxic action. To investigate such mechanism as well as gene regulation of this novel receptor, we have established an epithelial cell line, cDNA probes and anti-peptide antibodies. Thus, we are now able to explore further in vitro studies of neurotoxic actions of various environmental contaminants and drugs of abuse under controlled conditions. Since the choroid plexus is anatomically discriminated from other parts of the brain, it is easily obtained from the lateral ventricles. This anatomical characteristic helps us to study what we observe in vitro can take place in vivo.

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7. Giordano, J. and Hartig, P.R., Regulation of choroid plexus $5HT_{1C}$ receptor levels following lesion of serotonergic pathways by 5,7 dihydroxytryptamine (5,7 DHT) or 3,4 methylenedioxymethanphetamine (MDMA), manuscript in preparation.
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13. Kaufman, M.J., Hirata, F., Hartig, P.R. and Hoffman, B.J., Cyclic nucleotide modulation of $5HT_{1C}$ receptor mediated phosphoinositide turnover in porcine choroid plexus, and insensitivity to pertussis toxin, manuscript in preparation.
14. Kaufman, M.J. and Hartig, P.R., Efficacy of three families of serotonergic compounds on stimulation of $5HT_{1C}$ receptor mediated phosphoinositide hydrolysis in porcine choroid plexus, manuscript in preparation.

Abstracts:

1. Kaufman, M.J., Hirata, F., Hartig, P.R., Hoffman, B.J. (1989) Serotonin $5HT_{1C}$ receptor mediates cyclic GMP formation via arachidonic acid metabolites. Soc. Neurosci. Abstr. **15**: in press.

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